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14. ABSTRACT:

The purpose of this project is to determine the role of FGF receptor 1 in reactive stroma during prostate tumorigenesis. We are using a novel approach to target transgene expression specifically to the reactive stroma of experimental prostate cancer. We are placing an inducible Cre recombinase into the FAP gene locus to target expression to reactive stroma. We will cross this mouse with Fgfr1flox mice (LoxP sites flanking FGF receptor 1 alleles).

These mice will be crossed with TRAMP mice (prostate cancer model). Induced expression of Cre at sites of reactive stroma generated in the cancer foci will function to excise the FGF receptor 1 alleles and create a conditional knockout mouse. Progression of tumorigenesis in this line of knockout mice will be compared to heterozygous and wild type controls. Progress has been made in each Task. We have completed all cloning steps and acquired all reagents. We have rederived the Fgfr1flox and have crossed it into the appropriate backgrounds. We have completed crossing the TRAMP mice with the Fgfr1flox mice. This study will pinpoint the role of FGF receptor 1 in reactive stroma promotion of prostate cancer progression.

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Introduction:

Reactive stroma is generated in response to the development of carcinoma foci in the major human cancers. Reactive stroma is composed of both fibroblasts and myofibroblasts, with myofibroblasts evolving as a key cell type as cancer progresses. Reactive stroma exhibits elevated expression of several growth factors known to regulate wound repair and angiogenesis. Among these is fibroblast growth factor-2 (FGF-2), the focus of our project. Moreover, reactive stroma expresses matrix remodeling activity and synthesis of key matrix proteins including the collagens. Considerable evidence implicates elevated carcinoma cell expression of TGF-β1 as a principle inducer and regulator of reactive stroma. TGF-β1 induces most fibroblasts to a myofibroblast phenotype and stimulates expression of key growth factors including FGF-2 and connective tissue growth factor (CTGF). Each of these factors regulates reactive stroma biology, matrix remodeling, and stimulate rate of angiogenesis. In addition, considerable evidence shows that signaling pathways and biological responses between TGF-β1, CTGF, and FGF-2 are inter-regulatory. The formation of reactive stroma seems to be a predictable wound-repair type of response. As predicted, reactive stroma functions to return tissue to a state of normal homeostasis. Accordingly, the components of the normal stromal compartment have evolved with an inherent plasticity to respond rapidly to emerging situations. It is no wonder, then, that so many integrated regulatory components are housed in the stromal compartment. Along with stromal cells and matrix, the vasculature, the nerves, and immune components are all components of the stromal compartment (1, 2). Our recent work has shown the importance of TGF-β and CTGF signaling in the reactive stroma associated with prostate cancer (3-5). Less well understood is the role of FGF-2 signaling in the reactive stroma associated with prostate cancer and in transition of the epithelial carcinoma cell to a malignant phenotype. Our data to date shows that TGF-β1 regulates FGF-2 expression and release from prostate stromal cells. The role of FGF-2 signaling in the reactive stromal compartment of prostate cancer is unknown. Our project is based on the generation of a novel mouse model to knock out FGF-2 signaling in the reactive stromal compartment in order to address specific mechanisms and pinpoint those biologies specifically regulated by FGF-2 signaling. The expression of fibroblast activation protein (FAP) in the adult is restricted to reactive stromal cells (6, 7). Accordingly, we are using the FAP gene to specifically target the expression of an inducible Cre recombinase to reactive stromal cells. To accomplish this, we have proposed three Specific Aims and Tasks that will culminate in a conditional knock out of the FGF receptor 1 gene (cognate receptor for FGF-2) in the reactive stroma tumor microenvironment of the TRAMP mouse model for prostate cancer.

Body:

Task 1 will knock-in DNA encoding the Mifepristone (RU 486) inducible Cre recombinase (CrePR1) into the fibroblast activation protein (FAP) locus.

FAP expression is restricted to the mesenchyme during development and, in adult tissues, to reactive stroma fibroblasts during wound repair and in stromal responses to epithelial cancers. To specifically regulate FGF-2 signaling in the reactive stromal compartment of an experimental prostate cancer, we have proposed the use of the FAP gene locus. It is our intent to use the FAP promoter and regulatory elements to target a Mifepristone-regulated Cre recombinase (CrePR1) expression specifically to reactive stroma. The purpose of Task 1 is to generate this mouse. The use of an inducible Cre recombinase (Mifepristone) was proposed in order to selectively regulate expression at sites of reactive stroma formation in adult animals. This should, therefore, allow for expression of CrePR1 in specifically in prostate cancer reactive stroma, once this mouse has been crossed with the TRAMP mice (Task 3).

To utilize the FAP promoter, three possible approaches can be used. One approach is to use the FAP promoter to generate a construct containing the gene of choice and use this construct to generate a transgenic mouse. Since the FAP promoter is not fully understood and key regulatory elements have not yet been deduced, this is a somewhat risky approach at the present time. A second approach would be to knock in the inducible Cre recombinase into the actual native FAP gene locus. This is the approach that was originally proposed in the application. As discussed and disclosed in the previous progress report, we initiated this project based on the knockin approach, but have switched to using BAC clones. The primary issue is one of silencing the endogenous FAP locus, which would likely occur with a knockin approach. More recent data indicates that FAP is important to cancer progression and knocking out this gene, as a component of the knockin strategy would complicate the interpretation of the data. Accordingly, we have chosen to use a bacterial artificial chromosome (BAC) that contains the entire FAP mouse gene to knockin the inducible Cre recombinase (just downstream of the start site) through homologous recombination in bacteria, and then use this BAC clone to generate a transgenic mouse (FAP-CrePR1). A FAP-CrePR1 mouse generated in this manner will express inducible Cre recombinase under control of the full length, native FAP promoter and enhancer elements without disruption of the endogenous FAP gene and with no overexpression of additional FAP protein.

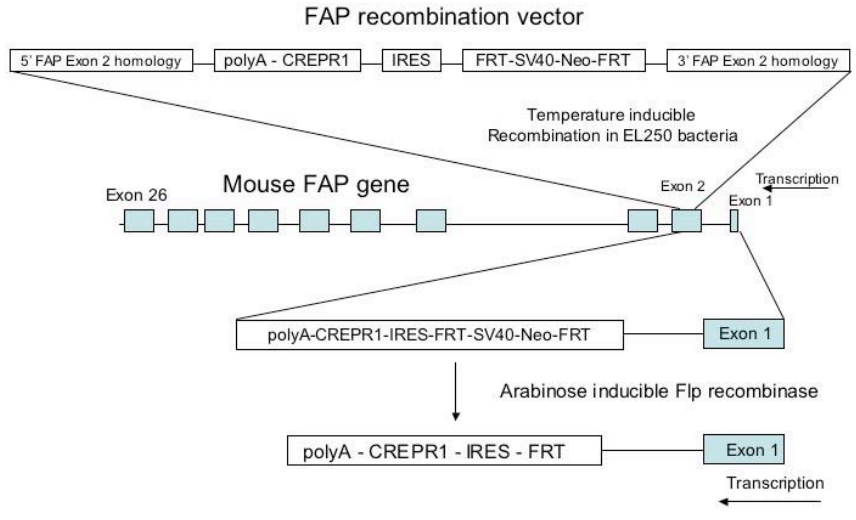
During the last progress period, we have finished all the cloning steps and are now conducting the homologous recombination steps (See Figures 1 and 2). Initially, we isolated and verified a BAC clone that contained the entire murine FAP gene (clone RP23-161B24). Next, the recombination vector was made by cloning an SV40 driven Neo gene flanked by Flp recombinase sites, followed by an IRES and then by an inducible (by RU486) CrePR1 followed by a polyA sequence. The Flp sites were engineered in to allow for removal of the Neo selection marker after selection and just prior to use of the construct in making

the transgenic mouse. These sequences were then flanked by 5' and 3" homology arms of 55 base pairs that were homologous to regions in the 2nd exon of the mouse FAP gene. This cloning strategy was complicated since endogenous FAP is transcribed from the anti-sense strand. Accordingly, after each cloning step the resulting construct was screened and fully sequenced in order to confirm that the CrePR1 when inserted into the FAP locus would be driven in the anti-sense direction. This has taken considerable time to establish fidelity and proper orientation of the construct, with some of the steps taking longer to complete than anticipated. This multistep cloning has now been completed and sequence integrity and orientation has been confirmed through multiple rounds of sequencing. A map of the final construct used for recombination is shown in Figure 2. All of the recombineering reagents were received from Neal Copeland and we are now recombining the IRES CrePR1 into the FAP BAC DNA in bacterial (EL250 cells) cultures. As of this report, we anticipate providing the recombined CrePR1 engineered FAP gene to our Genetically Engineered Mouse (GEM) Core laboratory for generation of the

Figure 1: Progress and Plans:

Task 1:	Isolation of a BAC clone (RP23-161B24) containing full length mouse FAP gene.
	Cloning SV40 driven Neo flanked by Flp recombinase sites
	Cloning in IRES element
	Cloning in CREPR1
	Cloning in poly A sequence
	Cloning in flanking FAP sequences (FAP gene Exon 2)
	Sequencing for proper recombination (FAP is transcribed off the antisense strand).
	Received all recombineering reagents (Neal Copeland)
	Recombining the IRES-CREPR1 into FAP DNA (double selection - chloramphenicol and kanamycin)
	Remove SV40/Neo (Flp recombinase - arabinose induced) (screen for kanamycin sensitive)
	Purify DNA and cut out FAP DNA and provide to GEM Core

Figure 2: Vector construction and recombination



transgenic mouse in the next month. We anticipate the next year of support to focus on Task 2 and 3.

Alternative Approaches: From the recombined BAC, we also plan to recombine out a fragment that contains 11 kb of the FAP upstream promoter, the Cre(PR1)-polyA and 2 kb of downstream FAP gene sequence. This fragment will be recombined out into a plasmid and purified. We plan to submit this to the GEM facility as well to produce an additional transgenic mouse as a backup plan should we have any problems with the full length BAC FAP recombined gene integrating into the transgenic mouse genome or with germ line transmission.

Task 2, is to cross these mice with a EF-1□ / lox stop cassette / FGFR1□ (dominant negative FGF receptor type I-myc tag) to create a FAP(CrePR1) / lox(stop) FGFR1□ bigenic animal. **Modification approved in the previous Progress Report:** The *Fgfr1*^{flox} mouse will be used instead of the FGFR1□ mouse for Task 2 in order to produce a conditional knockout.

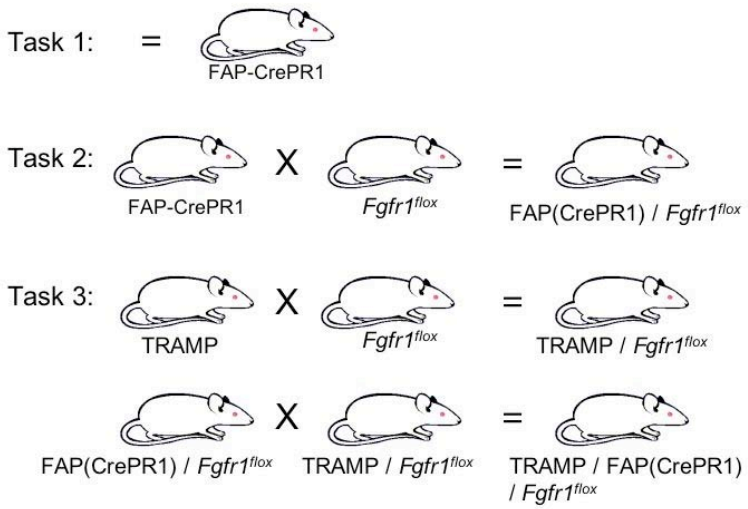
Our project is based on the hypothesis that FGF receptor I signaling is required in the reactive stromal compartment for prostate cancer progression. To address this directly, the overall goal of Task 2 is to knockdown or knockout expression of FGF receptor I at sites of reactive stroma. As we have reported previously, reactive stroma is tumor promoting and FGF receptor I signaling is likely a key mediator of this tumor-promoting biology (1, 2, 8). Accordingly, attenuation of FGF receptor I signaling should inhibit reactive stroma formation, which should inhibit tumor progression. As disclosed and approved in our last Progress Report, instead of expressing the lox(stop) FGFR1□ dominant negative to attenuate (knockdown) native gene expression, we have decided to use a recently generated mouse that contains floxed alleles of the FGF receptor I (*Fgfr1*^{flox} mice) (9) to fully knockout signaling. The heterozygous and homozygous *Fgfr1*^{flox} mice are phenotypically identical to wild type mice, making this model an excellent choice for generating a conditional knockout. Use of this mouse crossed with the FAP-CREPR1 mice manufactured in Task I will therefore produce a mouse that is both a conditional and inducible FGF receptor I knockout in cancer-associated reactive stroma, but yet retains expression of native FAP. Data generated from a conditional FGF-receptor I knockout mouse will be much preferred as compared with data from a FGF-receptor I attenuated (knockdown) mouse (FGFR1□). Hence we have proceeded in this direction. The *Fgfr1*^{flox} mice (ICR strain) was requested and provided to us by Juha Partanen (University of Helsinki, Finland). During the initial progress period we rederived these mice via embryo transfer and we now have the pathogen free colony in our TMF facility. In the last progress period we have bred this line (ICR strain) into the FVB and C57BL/6 background with now 8 sequential generations of breeding completed into each background. Statistically, this has yielded *Fgfr1*^{flox} mice now in both the FVB and C57BL/6 background having less than 0.39% ICR background. This background will be optimal for crossing with the FAP-CrePR1 mice generated in Task 1. This will then create the bigenic

FAP(CrePR1) / *Fgfr1*^{flox} (FVB) mouse line (heterozygous floxed FGFR1 allele). This step will complete Task 2. Figure 3 below shows the lines of mice and crosses to be made for each Task. In addition to the proposed experiments for Task 2, we have also generated a fibroblast cell line from the *Fgfr1*^{flox} mouse line peritoneum to use for testing.

Task 3, is to cross this bigenic animal with TRAMP mice. The TRAMP / FAP(CrePR1) / lox(stop) FGFR1 bigenic cross should exhibit RU 486 regulated expression of the dominant negative FGF receptor I transgene in TRAMP reactive stroma. **Modification approved in the previous Progress Report:** The resulting cross will produce the FAP(CrePR1) / *Fgfr1*^{flox} / TRAMP mouse that will result in RU 468 regulated FGF receptor I knockout in cancer associated reactive stroma (see Figure 3).

The TRAMP mouse acquires prostate cancer due to probasin (prostate specific) driven expression of SV40 large T antigen in prostate epithelial cells. Rapid tumors occur when female TRAMP mice in the C57BL/6 background are crossed with male FVB breeders. Accordingly, to fully analyze the effects of FGF receptor 1 signaling in reactive stroma, the first step of Task 3 was to cross homozygous TRAMP mouse with the homozygous *Fgfr1*^{flox} (C57BL/6 background) line generated in Task 2. This step has been completed. Inbreeding of this line has yielded the TRAMP/ *Fgfr1*^{flox} line of mice in the C57BL/6 background and heterozygous for floxed FGFR1 alleles and homozygous for TRAMP (first step in Task 3, see Figure 3 below). The second and final step of Task 3 will be to cross these TRAMP/ *Fgfr1*^{flox} (C57BL/6) mice with the FAP(CrePR1)/ *Fgfr1*^{flox} (FVB) mouse (also heterozygous for floxed FGFR1 alleles) generated in Task 2. This cross will yield [TRAMP/ *Fgfr1*^{flox} (C57BL/6)]/[FAP(CrePR1)/ *Fgfr1*^{flox} (FVB)] mice (Figure 3). All the experimental mice and controls for the project will be generated from this crossing and progeny screened for the FAP(CrePR1) transgene. We expect that approximately 25% of the progeny will be homozygous for the floxed FGFR1 alleles, which will be the conditional knockout experimental animals. Both FGFR1 alleles will be knocked out

Figure 3: Mouse lines generated in each Task



specifically at reactive stroma sites via Mifepristone-induction of Cre recombinase activity and removal of floxed alleles in reactive stromal cells. Controls will include the 50% of the FAP(CrePR1) transgenic mice will be heterozygous for the floxed FGFR1 alleles and 25% will have wild type FGFR1 alleles. All mice will receive Mifepristone and tumors will be evaluated as originally proposed. FAP should be expressed at the site of reactive stroma formation in the developing TRAMP tumor. Administration of Mifepristone should up regulate Cre recombinase specifically at these sites and the Cre should excise both FGFR1 alleles in the homozygous mice, creating a conditional knockout in tumor associated reactive stroma. Results will be compared to the TRAMP mice having heterozygous and wild type FGFR1 alleles and hence, functioning FGFR1. When exposed to Cre, a single allele knockout (heterozygous mice for floxed FGFR1 alleles) showed phenotypes similar to wild type controls in the previous studies of Partanen, which focused on developing mid- and hindbrain (9). The heterozygous and wild type mice are expected to have similar control phenotypes. The completion of Task 3 will produce data that will directly address the central hypothesis.

Key Research Accomplishments:

- Acquisition of BAC (clone RP23-161B24) containing the mouse FAP gene. Verification.
- Acquisition and subcloning of Mifepristone inducible Cre recombinase (CrePR1). Verification of sequence.
- Acquisition and subcloning of an SV40 Neo selection cassette flanked with Flp recombinase sites. Sequence verification.
- Construction and subcloning of a downstream IRES element. Sequence verification.
- Construction and subcloning of a downstream CrePR1. Sequence verification.
- Addition of a poly A tail downstream of CrePR1. Sequence verification.
- Step-wise construction and subcloning of flanking upstream and downstream 55 bp FAP gene sequence in reverse orientation (homologous to Exon 2 region of FAP gene). Sequence verification at each step for proper reverse orientation.
- Acquisition of EL250 cells containing an arabinose inducible *flpe* gene and all recombineering reagents.
- Acquisition of the *Fgfr1*^{flox} mice (ICR background) and confirmation of floxed alleles.
- Rederivation of the *Fgfr1*^{flox} mice by embryo transfer and initiation of *Fgfr1*^{flox} mice (ICR background) in TMF pathogen free facility.
- Acquisition of homozygous TRAMP mice and initiation of colony in TMF facility. Ready for crossing in Task 3.
- 8 generations of crossing *Fgfr1*^{flox} mice into FVB background. Now 0.39% ICR background. Ready for crossing Task 2 and 3.

- 8 generations of crossing *Fgfr1*^{flox} mice into C57BL/6 background. Now 0.39% ICR background. Ready for crossing in Task 2 and 3.
- 6 generations of crossing *Fgfr1*^{flox} mice with C57BL/6 (4 generations) and then crossing with TRAMP (2 generations). Now at less that 2% ICR background.

Reportable Outcomes:

During the last Progress Period I provided an invited Chapter on the biology of reactive stroma in cancer progression that discusses the role of FGF-2 signaling. This will be included as a chapter in the Textbook entitled: "Transforming Growth Factor-beta in Cancer Therapy" edited by Sonia B. Jakowlew at the NIH. My chapter title is: "Reactive Stroma and Evolution of Tumors: Integration of Transforming Growth Factor-□, Connective Tissue Growth Factor, and Fibroblast Growth Factor-2 Activities". This is both relevant to and supported by this project as this chapter discusses use of the FAP gene for targeting and the targeting of the FGF-2 signaling axis as a putative therapeutic. The chapter is 64 pages in length so it was not attached. It can be downloaded from the URL: https://bigfile.bcm.tmc.edu/outbound/drowley/RSChapter5fig%20copy.doc

- I will be happy to provide you with the chapter upon request.
- We have also published a manuscript in the last Progress Period that addresses the role of CTGF in prostate reactive stroma biology (5). This work is related to the present project as we propose that TGF-□ coordinately regulates expression and signaling of both FGF-2 and CTGF in reactive stroma. This paper represents the CTGF arm of this regulation and the present project represents the FGF-2 signaling arm. The reprint is attached. Title: "Stromal expression of connective tissue growth factor promotes angiogenesis and prostate cancer tumorigenesis".

Conclusions:

This study was designed to address the role of FGF receptor 1 signaling in the reactive stroma of prostate cancer in an experimental mouse model. Targeting gene expression specifically to sites of reactive stroma is a key goal of this project. The use of the FAP locus to accomplish this is a novel concept and generation of the FAP(CrePR1) mouse will be a resource for all investigators who study reactive stroma tumor microenvironment, since FAP gene expression is observed in reactive stroma of all the major adenocarcinomas.

The completed research to date is on track with what we have proposed as the major goals of the study and in the Statement of Work. We have acquired all the materials, clones and lines of cells and mice needed and have rederived them where necessary. Although it has take longer than expected, the cloning and construction of the inducible CrePR1 knockin vector is now complete. We have also completed the crossing of mice into the appropriate genetic background, as this is a key aspect of this project. We now anticipate no significant problems or time delays in completing the study as proposed. We

anticipate the next progress period will be spent crossing animals and assessing phenotypes.

This project represents the first time that a transgene will have been expressed specifically in the reactive stroma compartment of a tumor mouse model and represents the first time a gene will be conditionally knocked out in this compartment. Accordingly, this allows us for the first time to be able to study the significance of specific gene expression in the tumor microenvironment. This, in turn, will allow us to address the complex biology of this microenvironment in terms of a targeted therapeutic. Moreover, the FAP-CrePR1 mouse will be a valuable reagent for other investigators using mouse models of cancer as the inducible Cre can be used to manipulate the expression of a transgene or to attenuate gene expression using floxed alleles of a gene of choice.

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Stromal Expression of Connective Tissue Growth Factor Promotes Angiogenesis and Prostate Cancer Tumorigenesis

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Abstract

Our previous studies have defined reactive stroma in human prostate cancer and have developed the differential reactive stroma (DRS) xenograft model to evaluate mechanisms of how reactive stroma promotes carcinoma tumorigenesis. Analysis of several normal human prostate stromal cell lines in the DRS model showed that some rapidly promoted LNCaP prostate carcinoma cell tumorigenesis and others had no effect. These differential effects were due, in part, to elevated angiogenesis and were transforming growth factor (TGF)-β1 mediated. The present study was conducted to identify and evaluate candidate genes expressed in prostate stromal cells responsible for this differential tumor-promoting activity. Differential cDNA microarray analyses showed that connective tissue growth factor (CTGF) was expressed at low levels in nontumor-promoting prostate stromal cells and was constitutively expressed in tumor-promoting prostate stromal cells. TGF-β1 stimulated CTGF message expression in nontumor-promoting prostate stromal cells. To evaluate the role of stromal-expressed CTGF in tumor progression, either engineered mouse prostate stromal fibroblasts expressing retroviral-introduced CTGF or 3T3 fibroblasts engineered with mifepristone-regulated CTGF were combined with LNCaP human prostate cancer cells in the DRS xenograft tumor model under different extracellular matrix conditions. Expression of CTGF in tumor-reactive stroma induced significant increases in microvessel density and xenograft tumor growth under several conditions tested. These data suggest that CTGF is a downstream mediator of TGF-β1 action in cancer-associated reactive stroma and is likely to be one of the key regulators of angiogenesis in the tumor-reactive stromal microenvironment. (Cancer Res 2005; 65(19): 8887-95)

Introduction

Our previous studies have characterized reactive stroma in human prostate cancer progression and have developed the differential reactive stroma (DRS) xenograft model to address the role of reactive stroma in experimental prostate tumorigenesis. These studies have shown that reactive stroma initiates during prostatic intraepithelial neoplasia, exhibits a myofibroblast wound repair stromal phenotype, is tumor promoting, and is mediated, in part, by transforming growth factor (TGF)-β1 action (1–3). Our studies have also shown that reactive stroma was essential for inducing early angiogenesis and acted to stimulate both the incidence and rate of LNCaP prostate cancer cell tumorigenesis in DRS model xenografts (2). These studies showed that differential LNCaP tumor progression is based on the type of stroma in the xenograft tumor and the stromal response to TGF-β1.

Connective tissue growth factor (CTGF) has emerged as a potent mediator of TGF-β1 action in wound repair stromal responses and in fibrosis disorders (4–6). CTGF is a member of the CCN gene family (for CTGF, Cyr61, and Nov; refs. 7–9). This family includes six structural and functional related proteins: CTGF (10, 11); cysteine-rich 61 (Cyr61; ref. 12); nephroblastoma overexpressed (NovH; ref. 13); and Wnt-1–induced signaling protein (WISP) 1, WISP2, and WISP3 (14). The CCN family members (excluding WISP2) share four conserved structural modules with sequence homologies similar to insulin-like growth factor–binding protein, von Willebrand factor, thrombospondin, and cysteine knot (8). CTGF message is potently stimulated by TGF-β1 (15–19) and likely mediates TGF-β1–induced collagen expression in wound repair fibroblasts (20). CTGF is expressed by several stromal cell types, including endothelial cells, fibroblasts, smooth muscle cells, and myofibroblasts, and some epithelial cell types in diverse tissues. Consistent with its role in connective tissue biology, CTGF enhances stromal extracellular matrix synthesis (16) and stimulates proliferation, cell adhesion, cell spreading, and chemotaxis of fibroblasts (10, 16, 21). CTGF was also shown to stimulate smooth muscle cell proliferation and migration (22). In addition, CTGF is a potent stimulator of endothelial cell adhesion, proliferation, migration, and angiogenesis *in vivo* (23–25). As might be predicted, CTGF is expressed in the reactive stromal compartment of several epithelial cancers, including mammary carcinoma, pancreatic cancers, and esophageal cancer (26–28). Expression of CTGF is also observed in several stromal cell disorders, including angiofibromas, infantile myofibromatosis, malignant hemangiopericytomas, fibrous histiocytomas, and chondrosarcomas (29, 30). Accordingly, CTGF is considered to be a profibrosis marker (31). Together, these findings suggest that CTGF is a key regulatory factor for stromal tissue biology in wound repair and cancer progression; however, this has not yet been tested *in vivo* using engineered stromal cells.

Expression of TGF-β1 is elevated in most epithelial carcinoma cells (32) and our previous studies have shown that TGF-β1 is a critical regulator of carcinoma-associated reactive stroma, angiogenesis, and reactive stroma promotion of tumor progression in LNCaP xenograft tumors (3). Because TGF-β1 stimulates CTGF expression in stromal cells (15), including human prostate stromal cells (19), CTGF has accordingly emerged as a candidate downstream effector of TGF-β1 action in reactive stroma.

The DRS model system was specifically developed to evaluate differential gene expression in the reactive stromal compartment

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in xenografts composed of tissue-specific cancer cells and coordinate stromal cells (2, 3). These studies showed that two different human prostate stromal cell lines, HTS-2T and HTS-40C, exhibited differential effects in reactive stroma-induced angiogenesis and tumorigenesis of LNCaP prostate cancer cells (2). The present study was conducted to assess candidate genes responsible for the differential functions. We report here that CTGF was differentially expressed in tumor-promoting prostate stromal cell lines and that CTGF expression is stimulated by TGF-β1 in prostate stromal cells. In addition, we show that overexpression of CTGF in engineered prostate stromal cells in the DRS LNCaP xenograft model resulted in significantly elevated angiogenesis and LNCaP tumorigenesis *in vivo*.

Materials and Methods

Cell lines. LNCaP human prostate carcinoma cells were purchased from American Type Culture Collection (ATCC, Manassas, VA) and maintained in RPMI 1640 (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS, Hyclone, Logan, UT), 100 units/mL penicillin, and 100 μg/mL streptomycin (Sigma, St. Louis, MO). The HTS-2T and HTS-40C normal human prostate stromal cell lines were established in our laboratory (2) and cultured in Bfs medium: DMEM (Invitrogen) supplemented with 5% FBS (Hyclone), 5% Nu serum (BD Biosciences, Bedford, MA), 0.5 μg/mL testosterone, 5 μg/mL insulin, 100 units/mL penicillin, and 100 μg/mL streptomycin (Sigma). The Phoenix E packaging cell line was received from ATCC (by permission from Dr. Gary Nolan, Stanford University, Stanford, CA) and maintained in DMEM with high glucose (Invitrogen) supplemented with 10% heat inactivated FBS (Hyclone), 2 mmol/L glutamine (Invitrogen), and antibiotics as described above.

The mouse prostate stromal cell line, C57B, was derived from an 8-week C57BL/6 male mouse. The ventral prostate was removed, cut into 1 mm^3 cubes, and placed in wells of a six-well culture plate in Bfs medium and cultured at 37°C with 5% CO_2. Monolayers of stromal cells extended from the explants and, at confluence, the explants were removed and stromal cells were continued in culture by routine serial passage. C57B cells were positive for androgen receptor, vimentin, and smooth muscle α-actin with low expression of calponin (data not shown), similar to human prostate stromal cell lines we have reported previously (2). C57B cells were used at passages 15 to 25 for all experiments.

The GeneSwitch-3T3 cell line expressing the GeneSwitch regulatory protein from the pSwitch vector was purchased from Invitrogen. GeneSwitch-3T3 cells and derivative engineered cell lines were maintained in DMEM (Invitrogen) supplemented with 10% FBS (Hyclone), 100 units/mL penicillin, 100 μg/mL streptomycin (Sigma), and Hygromycin B and/or Zeocin (Invitrogen) as described below.

cDNA microarray analysis. HTS-2T and HTS-40C cells were cultured in Bfs medium to 80% confluence. Total RNA was extracted from each cell line with RNA STAT-60 total RNA/mRNA isolation reagent (Tel-test, Inc., Friendswood, TX) following the instructions of the manufacturer. Microarray analysis was done using 30 μg of total RNA. The cDNA reverse transcription and fluorescent labeling reactions were carried out using Cy3-labeled nucleotides for control (HTS-2T) and Cy5-labeled nucleotides for experimental (HTS-40C) samples as described previously (33). A microarray chip carrying 6,000 human cDNAs obtained from Baylor Microarray Core Facility was used. The hybridized slide was scanned with an Axon 4000A dual-channel scanner (Axon Instruments, Foster City, CA) and the data was analyzed using Gene Pix v. 3.0 software package (Axon). Genes were considered up-regulated if the expression was changed at least 3-fold from the control. Data with low signal intensity, high background, and high variability were eliminated.

Reverse transcription-PCR. Differential expression of CTGF in HTS-2T and HTS-40C cells was assessed by reverse transcription -PCR (RT-PCR) analysis. HTS-2T and HTS-40C cells were cultured in Bfs medium to 80% confluence and total RNA was extracted with the RNeasy Miniprep kit (Qiagen, Inc., Valencia, CA). CTGF amplification with primer 5′-GGTTACCAATGACAACGCCT-3′ and primer 5′-TGCTCCTAAAGCCACACCTT-3′ were used to monitor CTGF expression, by using the TaqMan one-step RT-PCR kit (Applied Biosystems, Foster City, CA).

To determine the effects of TGF-β1 on CTGF expression, HTS-2T cells were cultured to 80% confluence, exposed to M_0 serum-free media (MCDB 110 supplemented with insulin, transferrin, and sodium selenite; Sigma Diagnostics) for 24 hours, followed by 100 pmol/L (2.5 ng/mL) porcine TGF-β1 (R&D Systems, Minneapolis, MN) or vehicle control in M_0 media treatment for an additional 24 hours before total RNA extraction as described above. 18S rRNA amplifications with 18S rRNA primers (provided in the TaqMan one-step RT-PCR kit) were used for total RNA loading control. RT-PCR reactions were carried out in 50 μL total volume with 80 ng of total RNA and 32 pmol of each primer. First-strand synthesis was done at 48°C for 30 minutes. For CTGF amplification, PCR cycles were run at 95°C for 15 seconds, 60°C for 2 minutes, for a total of 28 cycles. For 18S rRNA amplification, PCR cycles were run at 95°C for 15 seconds, 60°C for 1 minute, for a total of 20 cycles. The PCR products were electrophoresed through a 2% agarose gel, visualized with ethidium bromide, and photographed. A similar RT-PCR procedure was carried out to monitor CTGF expression in HTS-2T and HTS-40C cells, with a total RNA of 200 ng per reaction.

Retroviral infection. The pRc/CMV-CTGF plasmid containing human CTGF cDNA was a kind gift from Dr. Gary Grotendorst (Lovelace Respiratory Research Institute, Albuquerque, NM; ref. 16, 26). For the construction of pBMN-CTGF-I-enhanced green fluorescent protein (EGFP) vector for retroviral delivery of CTGF, the human CTGF cDNA coding sequence was excised with *Eco*RI from pRc/CMV-CTGF vector and ligated into the pBMN-I-EGFP retroviral vector kindly provided by Dr. Gary Nolan with the same restriction site. Clones were sequenced to ensure correct CTGF cDNA orientation and sequence.

The pBMN-CTGF-I-EGFP vector (bicistronic) or pBMN-I-EGFP control vector were transfected into Phoenix E cells with a calcium phosphate transfection kit (Invitrogen) following a modified protocol. In brief, Phoenix cells were seeded at 1.5×10^6 cells in a 6 cm culture plate 24 hours before transfection. For transfection, 10 μg of DNA and 61 μL of 2 mol/L $CaCl_2$ were brought to 0.5 mL with double-distilled water and added dropwise to 0.5 mL of 2× HBS, while aerating with a pipette, and followed by 30-minute incubation at room temperature to form fine precipitates. To Phoenix cells in 6 cm plates in 3 mL media, 2 μL of 50 mmol/L chloroquine stock were added. Five minutes later, DNA/$CaHPO_4$ precipitates were added dropwise, followed by overnight incubation at 37°C. Medium was replaced 24 hours after transfection and plates were incubated at 32°C. Virus in the supernatant from each retrovirus-producing line was collected 48 hours after transfection and filtered (0.45 μm). Three milliliters of viral supernatant with additional 5% FBS, 5% Nu serum (BD Biosciences), 0.5 μg/mL testosterone (Sigma), 5 μg/mL insulin, and 5 μg/mL polybrene was applied immediately to C57B prostate stromal cells at 60% to 80% confluence in T25 flask. Infection was carried out at 37°C. Viral supernatant was replaced with fresh Bfs medium 24 hours after infection. Expression of retroviral construct was confirmed by counting the percentage of green fluorescent (GFP positive) C56B cells per ×100 field. Infected cultures with a >90% green fluorescent cells per field were passaged and frozen (−80°C) in 4×10^6 cells/vial aliquots for use in DRS xenografts.

3T3 cell GeneSwitch system. The GeneSwitch system (Invitrogen) was used to engineer 3T3 fibroblast cells with mifepristone (RU 486) inducible expression of a V5-His tagged CTGF protein. GeneSwitch-3T3 cells expressing the GeneSwitch regulatory protein from the pSwitch vector were purchased from Invitrogen. For the construction of pGene CTGF-V5-His vector, the human CTGF cDNA was PCR amplified from pRc/CMV-CTGF with primers 5′-CTAGGATCCGCCCGCAGTGCC-3′ (*Bam*HI) and primer 5′-TCTCTGGGGCCCTGCCATGTCTCCGTACATCTTC-3′ (*Apa*I). PCR cycles were run at 95°C for 30 seconds, 60°C for 30 seconds, and 72°C for 80 seconds for a total of 20 cycles after first incubation at 95°C for 2 minutes. The PCR reaction was incubated at 72°C for another 10 minutes for final extension. PCR products were purified with QIAquick PCR purification kit (Qiagen). After digestion with *Bam*HI and limited digestion with *Apa*I (to avoid internal *Apa*I site along CTGF cDNA sequence), the 1.1 kb CTGF insert was

gel purified and cloned in frame into the pGene/V5-His A vector (Invitrogen). Fidelity was confirmed by sequence analysis. The pGene CTGF-V5-His vector or pGene/V5-His empty vector control was transfected into GeneSwitch-3T3 cell line (Invitrogen) with FuGENE 6 transfection reagent (Roche Diagnostics Corp., Indianapolis, IN), following the protocol of the manufacturer. Stable transfected GeneSwitch-3T3 cells were selected and maintained in media (as described previously) containing 50 μg/mL of Hygromycin B and 200 μg/mL of Zeocin. Mifepristone (100 pmol/L) was used to induce CTGF-V5-His fusion protein expression. Regulated expression was confirmed by Western blot analysis of secreted proteins.

To render engineered GeneSwitch-3T3 pGene CTGF-V5-His and GeneSwitch-3T3 pGene/V5-His cells less proliferative and less tumorigenic for use in the DRS xenograft model, the cells were irradiated with increasing doses of γ-irradiation. The γ-irradiation dosage of 800 rad was chosen for DRS xenograft tumor experiments because it resulted in viable cells with a low proliferation rate and high expression of mifepristone-inducible CTGF-V5-His protein *in vitro* (Western blot, data not shown).

Western blot analysis. For V5 Western blot, conditioned medium from GeneSwitch-3T3 pGene CTGF-V5-His cells induced with 100 pmol/L mifepristone (or vehicle control) was electrophoresed through a 12% SDS-PAGE gel. Proteins were transferred onto nitrocellulose membrane (Bio-Rad Laboratories, Hercules, CA) and incubated in PBS buffer with 5% nonfat milk at 4°C overnight. Mouse anti-V_5 monoclonal antibody (Invitrogen), diluted at 1:5,000, was used as primary antibody to detect the presence of CTGF-V5-His fusion protein, and incubated for 2 hours at room temperature. Secondary antibody was biotin-conjugated sheep anti-mouse IgG (Sigma), diluted at 1:1,000, and incubated for 1 hour at room temperature. A streptavidin-horseradish peroxidase conjugate (Amersham Pharmacia Biotech UK, Ltd., Buckinghamshire, United Kingdom) diluted at 1:1,000 was incubated for 30 minutes at room temperature. Protein bands were detected by incubation with ECL+ Western blotting detection system (Amersham Biosciences) for 5 minutes at room temperature followed by exposure to Hyperfilm ECL from Amersham Pharmacia Biotech.

For CTGF Western blot, C57B CTGF and control cells were grown in Bfs to 80% confluence, then switched to serum-free M_0 media for 2 days. The media were collected and concentrated 20-fold by Amicon Ultra 4 centrifugation (5000 MWCO; Millipore, Billerica, MA). The concentrated samples were electrophoresed through a 12% SDS-PAGE gel and proteins were transferred onto Immobilon-P (Millipore). The membrane was incubated in PBS buffer with 2.5% normal donkey serum at 4°C overnight. The immunoblot protocol was the same as above, except the primary antibody was goat anti-CTGF antibody L-20 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), diluted at 1:400, and secondary antibody was biotin-conjugated donkey anti-goat antibody (Jackson ImmunoResearch Laboratories Inc., West Grove, PA), diluted at 1:40,000.

Animals and preparation of differential reactive stromal xenografts. Athymic NCr-*nu/nu* male homozygous nude mice, 6 to 8 weeks of age, were purchased from Charles River Laboratories (Wilmington, MA). All experiments were in compliance with the NIH Guide for the Care and Use of Laboratory Animals and according to the institutional guidelines of Baylor College of Medicine.

DRS xenograft tumors were generated following procedures we have published previously (2, 3, 34). Briefly, frozen aliquots of LNCaP human prostate cancer cells (16×10^6) and the engineered stromal cells—C57B-CTGF (8×10^6 cells), C57B-control (8×10^6 cells), and γ-irradiated GeneSwitch-3T3 pGene CTGF-V5-His cells (4×10^6 cells)—were thawed in a 37°C water bath for 1 to 2 minutes and washed once with 10 mL RPMI supplied with 10% serum (for LNCaP cells) or with 10 mL DMEM supplied with 10% serum (for stromal cells) in 15 mL conical tubes. The cells were pelleted at $1,400 \times g$ for 2 minutes and resuspended in 6 mL RPMI 1640 with 10% FBS. The LNCaP cells were then combined with stromal cells, mixed well, and pelleted again at $1,400 \times g$ for 2 minutes. The supernatant was aspirated to either 300 μL (for Matrigel experiments) or 200 μL [for growth factor–reduced (GFR) matrix mixture experiments] and cells were resuspended in the remaining medium. Cells were incubated on ice for 1.5 minutes and then combined with either 0.5 mL of Matrigel (Becton Dickinson, Bedford, MA) or 0.6 mL of a GFR matrix mixture composed of a 1:1 ratio of neutralized Vitrogen 100 (99.9% collagen type I; Cohesion, Palo Alto, CA) and GFR Matrigel (Becton Dickinson). In all experiments, the final volume was 800 μL. The cell and matrix mixture was drawn into a 1 mL syringe fitted with a 20-gauge needle. After switching to a 25-gauge needle, 100 μL of the cell-matrix suspension was injected s.c. in each lateral flank of adult NCr-*nu/nu* male mice.

To induce expression of CTGF-V5-His, mice with DRS xenografts composed of LNCaP cells combined with γ-irradiated GeneSwitch-3T3 pGENE CTGF-V5-His cells received mifepristone (Sigma) or vehicle control (sesame seed oil; Sigma) at 0.5 mg/kg administered as 100 μL i.p. injections at the time of tumor injection and repeated every 48 hours until tumors were harvested. This mifepristone dose was based on protocols shown previously to induce consistent gene expression *in vivo* and had no affect on xenograft tumor weight or volume (data not shown). All mifepristone experiments are in accordance with our approved Animal Use Protocols and institutional guidelines of Baylor College of Medicine.

Tumors were collected at different time points between days 10 and 21 postinoculation. For the experimental sets of LNCaP cells combined with C57B-CTGF or C57B-control cells, the tumors were photographed *in situ* for GFP expression to confirm gene expression using a fluorescent dissecting microscope. The tumors were weighed, measured in three dimensions, and fixed in 4% paraformaldehyde (neutral buffered) at 4°C overnight, washed three times in PBS, and processed for paraffin embedding. Tumors were paraffin-embedded and 5 μm sections were cut and mounted onto ProbeOn Plus slides (Fisher Scientific, Pittsburgh, PA). Sections were either stained with H&E for histologic analysis or processed for immunohistochemistry.

Immunohistochemistry. Primary antibodies were as follows: anti-mouse CD31/platelet/endothelial cell adhesion molecule 1 antibody (rat monoclonal MEC13.3; BD PharMingen, San Diego, CA); anti-V5 mouse monoclonal antibody 46-0705 (Invitrogen); rabbit anti-GFP antibody A-11122 (Molecular Probes, Eugene, OR); goat anti-CTGF antibody L-20 (Santa Cruz). Secondary antibodies were as follows: biotin-conjugated goat anti-rat IgG (BD PharMingen) for CD31, biotin-conjugated Universal Secondary (Invitrogen) for V5, biotin-conjugated goat anti-rabbit IgG B8895 (Sigma) for GFP, and biotin-conjugated donkey anti-goat antibody (Jackson ImmunoResearch Laboratories). Specificity of each primary antibody has been evaluated previously (refs. 2, 3, 34; and unpublished data).

Immunostaining was done with the MicroProbe Staining System (Fisher Scientific) following our protocol published previously (2, 3, 34). Reagents formulated for use with capillary action systems were purchased from Open Biosystems (Huntsville, AL) and used according to the protocol of the manufacturer. In brief, tissues were deparaffinized using Auto Dewaxer and cleared with Auto Alcohol. Brigati's iodine and Auto Prep were used to improve tissue antigenicity. Antigen retrieval were used in CD31, V5, and CTGF staining. For CD31 staining, tissues were incubated in 0.1% trypsin (Zymed, South San Francisco, CA) for 10 minutes at 37°C; for V5 and CTGF staining, tissues were subjected to high-temperature-steamer treatment in 10 mmol/L sodium citrate buffer (pH 6.0) for 20 minutes. Goat anti-mouse Fab fragment (Jackson ImmunoResearch Laboratories) 1:65 was used for 30 minutes at 37°C for blocking before anti-V5 immunostaining. Sections were then incubated in protein blocker (for V5, CD31, and GFP) or 5% normal donkey serum in universal buffer (for CTGF). Primary antibodies were diluted and used under the following conditions: V5 (1:200), CD31 (1:50), GFP (1:200) in primary antibody diluent, and CTGF (1:100) in 5% normal donkey serum overnight at 4°C. Secondary antibodies were diluted and used under following conditions: biotin-conjugated universal secondary antibody for 4 minutes at 50°C; biotin-conjugated goat anti-rat IgG 1:100; biotin-conjugated goat anti-rabbit IgG 1:500; and biotin-conjugated donkey anti-goat antibody 1:200 for 45 minutes at 37°C. Tissues were treated with Auto Blocker to inhibit endogenous peroxidase activity. For detection, sections were incubated in RTU VectaStain Elite ABC reagent (Vector

Laboratories, Burlingame, CA) and then incubated in stable diaminobenzidine tetrahydrochloride twice for 3 minutes each at 50°C. Tissues were counterstained with Auto Hematoxylin for 30 seconds.

Microvessel density analysis. Analysis was done according to standard procedures we have published previously with DRS tumors (2, 3, 34). Tissue sections were stained for CD31 as described above. Sections were scanned at ×100, and five random areas per tumor section were selected. Vessels in these fields were counted (at ×400) by an observer blinded to experimental conditions. The average vessel count was determined for each specimen.

Statistical analysis. Tumors from each condition were analyzed, and average tumor weight and average microvessel counts were compared with these values from their matching control tumors for statistical relevance using the unpaired *t* test. Statistical analyses used GraphPad Prism for Macintosh version 3.0 (GraphPad Software, San Diego, CA). $P < 0.05$ was considered statistically significant.

Results

Differential expression of connective tissue growth factor in tumor-promoting human prostate stromal cell lines. Our previous studies using the DRS xenograft model showed that several human prostate stromal cell lines differentially promote LNCaP prostate cancer cell tumorigenesis (2). Stromal cell–promoted tumors exhibited a significantly elevated rate of angiogenesis and this was TGF-β1 regulated (2, 3). Notably, the HTS-40C and the HTS-2T human prostate stromal cell lines exhibited opposing effects. In two-way DRS xenografts constructed of cancer cells and stromal cells in the absence of extracellular matrix (Matrigel), the HTS-40C/LNCaP combinations resulted in a 65% tumor incidence, whereas HTS-2T/LNCaP combinations were nontumorigenic (0% tumor incidence; ref. 2). To address potential mechanisms, gene expression profiles in HTS-40C and HTS-2T stromal cells were compared using cDNA microarray analyses. This analysis showed that 12 previously characterized genes were elevated by 3- to 31-fold in the protumorigenic HTS-40C stromal cell line compared with HTS-2T. These genes are listed in Table 1. Expression of several of these genes is associated with reactive stroma that forms at sites of wound repair, microbial invasion, or carcinoma as we have reported previously (1, 32, 35). Of these, CTGF is a known inducer of angiogenesis (36), is TGF-β1 regulated in stromal cells (18, 37–39), and has been reported to directly enhance TGF-β1 receptor-ligand binding (40). Our microarray data suggested that CTGF message was 4.5-fold higher in HTS-40C cells compared with HTS-2T cells. Further analysis confirmed this with RT-PCR and showed that CTGF message expression was severalfold higher in HTS-40C cells relative to HTS-2T cells as shown in Fig. 1*A*.

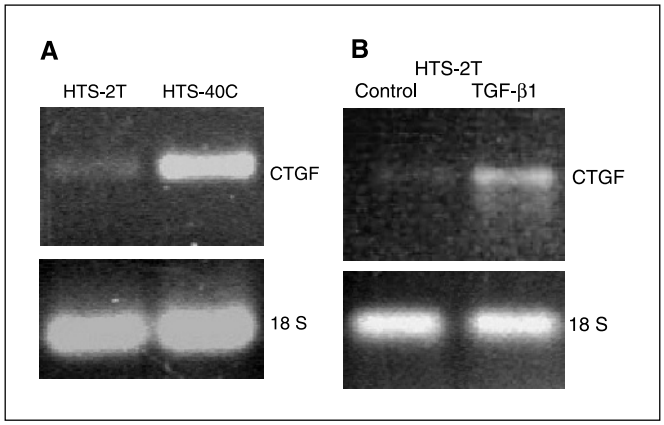

Figure 1. Expression of CTGF message in different prostate stromal cell lines. *A,* RT-PCR–amplified products from HTS-40C cells compared with HTS-2T cells. *B,* HTS-2T cells exposed to TGF-β1 (100 pmol/L) or vehicle control for 24 hours. In both cases, 18S rRNA amplifications were used as loading control.

Although the HTS-2T stromal cell line did not support LNCaP tumorigenesis in matrix-free conditions (two-way tumors), HTS-2T cells did promote LNCaP tumors (incidence, rate of tumorigenesis, and angiogenesis) when combined with Matrigel matrix in three-way DRS xenografts that are constructed with cancer cells, stromal cells, and Matrigel matrix (2, 3). Matrigel matrix is high in TGF-β1 and we have reported that inhibiting TGF-β1 activity in Matrigel lowers the rate of tumorigenesis and angiogenesis in three-way DRS tumors (3). Accordingly, we next determined whether TGF-β1 could induce CTGF expression in human prostate HTS-2T stromal cells. As shown in Fig. 1*B*, HTS-2T cells in control conditions exhibited low expression, whereas HTS-2T cultures exposed to TGF-β1 (100 pmol/L, 24 hours) exhibited elevated CTGF message expression. This is in agreement with previous reports showing TGF-β1 regulation of CTGF expression in other stromal cell lines (15, 19).

Table 1. Genes up-regulated in the HTS-40C cells compared with HTS-2T cells

40C/2T	Gene	UniGene no.	Accession no.	Gene description
31.333	*PLOD2*	Hs.477866	U84573	Procollagen-lysine, 2-oxoglutarate 5-dioxygenase 2
10.800	*TRAP1*	Hs.30345	U12595	Tumor necrosis factor receptor–associated protein 1
7.666	*TP53BP2*	Hs.523968	AI123916	Tumor protein p53-binding protein, 2
5.847	*ARF3*	Hs.119177	M74493	ADP ribosylation factor 3
4.899	*CFH*	Hs.363396	M12383	Complement protein H
4.637	*FMO*	Hs.132821*	AL021026	Flavin-containing monooxygenase
4.466	*CTGF*	Hs.75511	U14750	Connective tissue growth factor
3.841	*THBS1*	Hs.164226	NM_003246	Thrombospondin 1
3.574	*BRAP*	Hs.530940	AF035950	BRCA1-associated protein
3.152	*ADH1A*	Hs.368549	NM_000667	Alcohol dehydrogenase 1A (class I), α polypeptide
3.130	*PTSG1*	Hs.201978	U63846	Prostaglandin-endoperoxide synthase 1 (prostaglandin G/H synthase and cyclooxygenase)
3.083	*PTX3*	Hs.546280	M31166	Pentraxin-related gene, rapidly induced by interleukin-1β

*Retired UniGene number without concise replacement.

Expression of connective tissue growth factor in prostate stromal cells promotes angiogenesis and LNCaP tumorigenesis. A construct containing the full-length human CTGF cDNA (kindly provided by Dr. Gary Grotendorst) was used to construct a bicistronic retroviral vector (pBMN-CTGF-I-EGFP) containing CTGF followed by an IRES and EGFP for detection of expression. Either vector control (pBMN-I-EGFP) or the CTGF-containing retrovirus preparations were used to infect the mouse prostate stromal cell line (C57B) and cells were analyzed for fluorescence 48 hours later as described in Materials and Methods. Figure 2*A* shows infected and EGFP-expressing C57B stromal cells before use in the DRS xenograft. C57B cells routinely exhibited a 90% infectivity rate or higher (data not shown). Western blot analysis showed overexpression of the mature form of CTGF (~38 kDa) in the experimental cell conditioned medium and low endogenous levels in the control infected cultures (Fig. 2*B*). Shorter fragments were also observed (Fig. 2*B*, *band A* and *band B*), which have been reported in the conditioned media of CTFG-secreting cells by others (41).

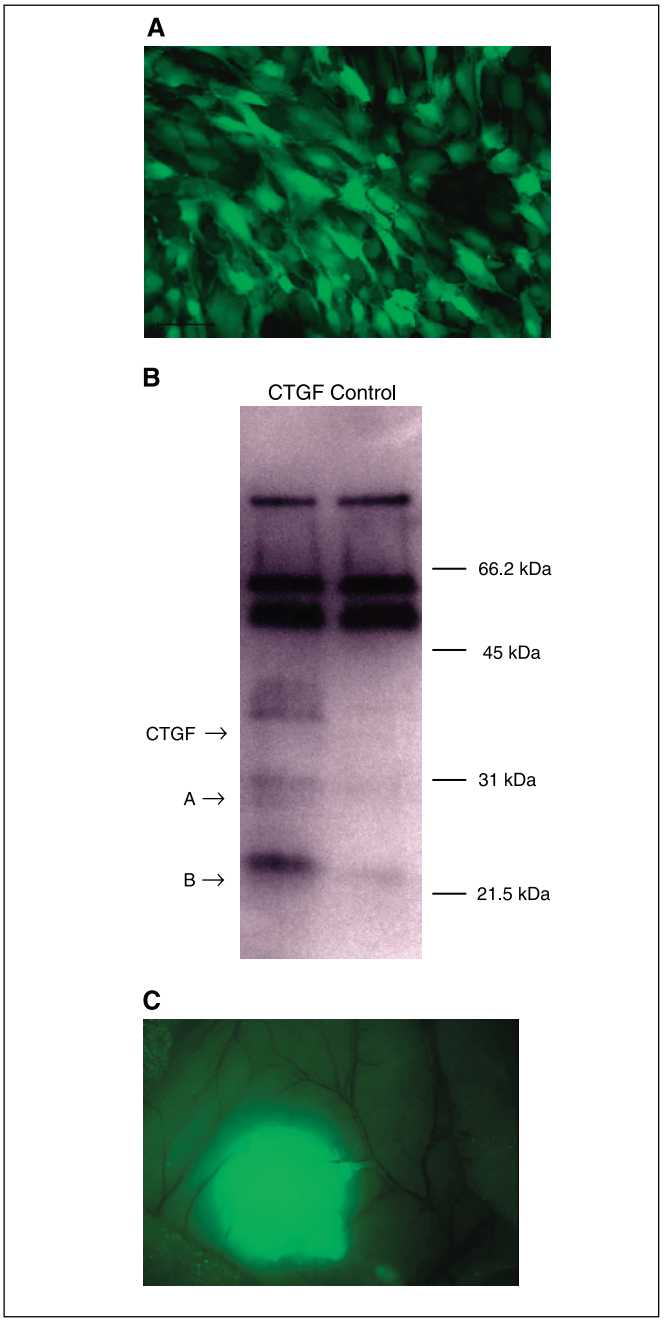

Figure 2. Transgene expression in retroviral-infected C57B prostate stromal cells and DRS tumors. *A,* GFP fluorescence of retroviral (pBMN-CTGF-I-EGFP) infected C57B cells *in vitro*. *Bar*, 100 μm. *B,* Western blot analysis of CTGF protein in conditioned media of pBMN-CTGF-I-EGFP–infected C57B cells (*CTGF*) compared with pBMN-I-EGFP control vector infected cells (*Control*). *C,* GFP fluorescence of tumors *in situ*. An incision was made in the skin immediately adjacent to the s.c. tumor. The skin flap was turned back and photographed with a fluorescent dissecting microscope.

To evaluate the effects of CTGF expression from prostate stromal cells in three-way LNCaP tumors in nude mice, we inoculated cell combinations in either complete Matrigel or a modified matrix composed of a 1:1 mix of GFR Matrigel together with neutralized Vitrogen 100 collagen type I (GFR Matrigel/Vitrogen) to reduce bioactive factors in the matrix component. S.c. three-way DRS xenograft tumors were constructed in male nude mice using 2×10^6 LNCaP cells, and 1×10^6 control C57B (EGFP-expressing vector only) or CTGF-expressing C57B prostate stromal cells and the different Matrigel matrix preparations as described in Materials and Methods. Tumors were harvested at day 13 postinoculation because our previous studies have shown that day 10 to day 14 postinoculation is the optimal time frame to assess initial rate of angiogenesis and tumorigenesis (2, 3, 34). It should be noted that control or CTGF-transduced C57B cells inoculated alone or with matrix were nontumorigenic (data not shown) similar to our previous report (2). As shown in Fig. 2*C*, tumors were fluorescent *in situ* before removal. This confirmed transgene expression and viability of the engineered C57B stromal cells in the tumor xenograft.

Tumors exhibited a typical arrangement of LNCaP carcinoma cell clusters, surrounded by stromal cells, matrix, and vessels as shown in Fig. 3*A* and *B*, similar to what we have reported previously (2). There were no particular differences in histology or ratio of carcinoma to stromal cells in experimental tumors compared with control tumors. Prostate stromal cells engineered with the CTGF transgene in tumors were positive for both EGFP (Fig. 3*C*) and CTGF (Fig. 3*D*) proteins, and were immediately adjacent to clusters of LNCaP carcinoma cells. Immunostaining for CD31 as an endothelial marker showed an obvious difference in vessels. The density of CD31-positive microvessels in CTGF-expressing xenografts (Fig. 3*F*) seemed higher compared with control xenografts (Fig. 3*E*). Microvessel counts confirmed this. In complete Matrigel conditions, LNCaP xenograft tumors constructed with CTGF-expressing prostate stromal cells exhibited a microvessel density of 10.60 ± 1.35 compared with 6.16 ± 1.60 in vector-only control tumors ($n = 25$ fields, five tumors each, mean $\pm$ SE, $P < 0.05$; Fig. 4*A*). This represented a 72% increase in vessel density in the stromal CTGF-expressing tumors. The increase in vessel density correlated with elevated tumor mass. The mean wet weight of stromal CTGF-expressing LNCaP tumors was 24.42 ± 0.76 mg compared with 18.08 ± 1.54 mg ($n = 5$, mean $\pm$ SE, $P < 0.01$; Fig. 4*B*) in control tumors, indicating that stromal CTGF expression produced a 35% increase in tumor mass when xenografts are constructed in complete Matrigel conditions.

Significant differences in angiogenesis were even more pronounced in the low growth factor–modified matrix (GFR Matrigel/Vitrogen 100) conditions. CTGF-expressing tumors exhibited an average microvessel density of 10.10 ± 1.73

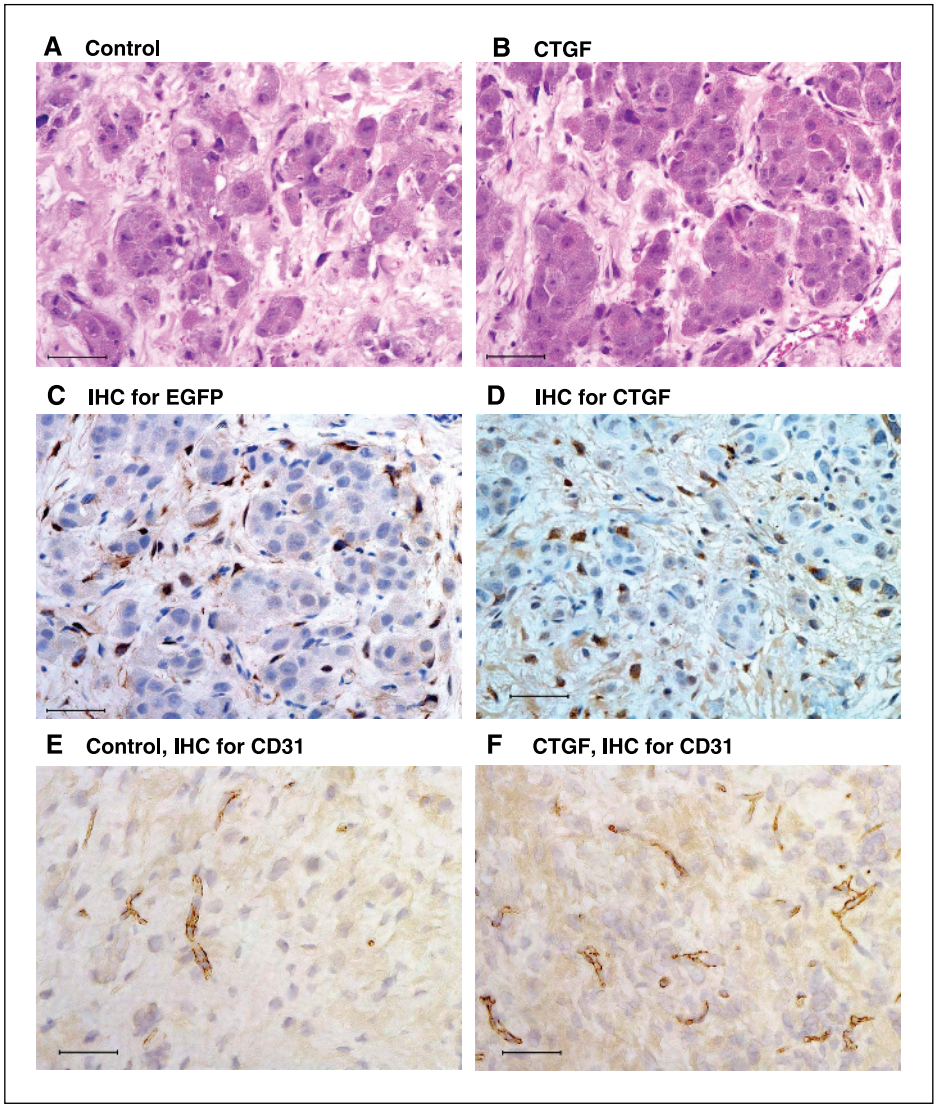

Figure 3. Histologic analysis of three-way LNCaP DRS xenograft tumors constructed with control or CTGF-expressing prostate stromal cells. *A* and *B,* the histology of DRS tumors generated from LNCaP cells combined with control C57B cells (*A*) or CTGF-expressing C57B cells (*B*). *C,* immunohistochemistry of EGFP expression in tumor stromal cells. *D,* immunohistochemistry of CTGF expression in tumor stromal cells. *E,* immunohistochemistry of CD31 expression in vessels from tumors constructed with control stromal cells. *F,* immunohistochemistry of CD31 expression in vessels of tumors constructed with CTGF-expressing stromal cells. *Bar*, 50 μm.

compared with 4.70 ± 1.00 in control tumors, representing a 115% increase over control (n = 30, from six tumors in each condition, mean ± SE, P < 0.01; Fig. 4*C*). The stromal CTGF-expressing LNCaP tumors constructed in the GFR-modified matrix showed an average wet weight of 17.58 ± 0.60 mg compared with 12.97 ± 0.71 mg in control tumors (n = 18 in the CTGF experimental and n = 17 in the control, mean ± SE, P < 0.0001; Fig. 4*D*), representing a 36% increase in tumor mass.

Regulated expression of CTGF-V5-His in 3T3 fibroblasts promotes LNCaP tumorigenesis. To confirm and extend the findings with retroviral transduced C57B cells, the GeneSwitch System (Invitrogen) was used to engineer 3T3 stromal cell lines with mifepristone-regulated expression of an epitope-tagged CTGF-V5-His (fusion protein). Cultures at 80% to 100% confluence were induced with 100 pmol/L mifepristone for 24 to 48 hours. Western blot analysis for the V5 epitope showed an inducible 41 kDa CTGF-V5-His band in the conditioned media (Fig. 5*A*). DRS xenograft tumors were generated in nude mice using 2 × 10^6 LNCaP cells combined with γ-irradiated 5 × 10^5 GeneSwitch-3T3 pGene CTGF-V5-His cells and complete Matrigel (three-way DRS xenograft conditions). Irradiated engineered 3T3 cells (800 rad) were used because these cells remain viable, exhibit regulated transgene expression, and have a low proliferative rate relative to wild-type NIH 3T3 cells. Mice were given mifepristone or vehicle i.p. every 48 hours as described in Materials and Methods. Our previous studies have shown that this protocol of mifepristone treatment has no ill effect on nude mice and does not affect control tumor biology (2, 34). Resulting tumors were harvested 10 days postinoculation. Immunohistochemistry showed tightly regulated CTFG-V5-His protein expression *in vivo* (Fig. 5*B*). No expression was noted in tumors derived from vehicle control-treated animals (Fig. 5*C*). Tumors exhibited a typical carcinoma phenotype similar to the LNCaP/C57B combinations, although the tumors were considerably more heterogeneous with more focal nodules of carcinoma and other areas that seemed to have little carcinoma growth. There was, however, no apparent difference in histopathology noted between vehicle control and mifepristone-treated animals. LNCaP DRS tumors from mifepristone-treated animals exhibited a 25% average increase in wet weight as shown in Fig. 5*D*. The mean weight of control tumors was 17.91 ± 1.04 mg, whereas tumors from mifepristone-treated animals averaged 22.41 ± 1.76 mg (P < 0.05,

n = 12 tumors each). The tumors exhibited a very heterogeneous density of microvessels, as might be expected, due to the nodular and heterogeneous histopathology. This was obvious at low-power observation (data not shown). The heterogeneous nature of the vessel density patterns in these tumors was not compatible with the microvessel-counting protocol (see Materials and Methods) as the accuracy of this method is dependent on uniform vessel distribution. Accordingly, no attempt was made to quantitate microvessel density in these tumors as these data would not be accurate.

Discussion

To date, no effective approach exists to manipulate overexpression of a transgene in the stromal compartment in a tissue-specific manner *in situ*. Accordingly, we have used the DRS xenograft tumor approach to test the biological consequences of differential transgene expression in the reactive stroma compartment of an experimental human tumor in a nude mouse host. Our previous studies have shown that use of different human prostate stromal cell lines result in vast differences in LNCaP tumorigenesis *in vivo* (2). Furthermore, we have shown that the endogenous TGF-β1 activity in complete Matrigel is responsible for this difference in both angiogenesis and tumorigenesis (3). Our current study shows that CTGF may mediate TGF-β1 actions in the prostate stromal cells. Expression of a CTGF transgene in the reactive stromal compartment of LNCaP DRS xenograft tumors resulted in enhanced tumorigenesis that was correlated with a more rapid rate of angiogenesis. We conclude from these data that CTGF may be an important regulator of tumor-reactive stroma and angiogenesis.

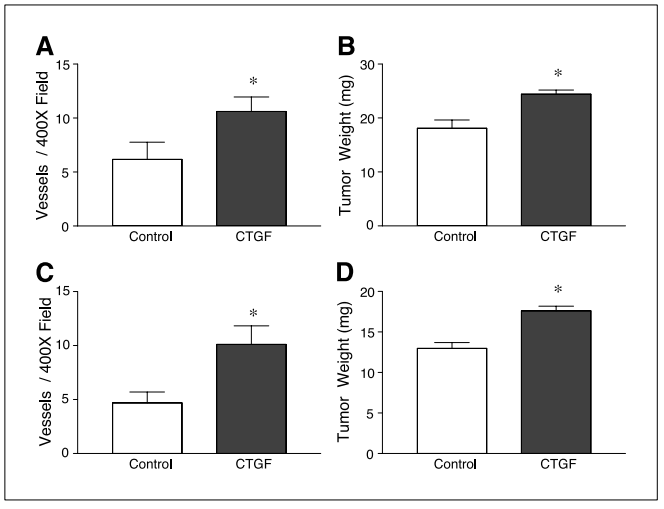

Figure 4. Stromal expression of CTGF stimulates microvessel density and tumor weight in three-way LNCaP DRS tumors constructed in different matrix preparations. *A* and *B,* microvessel densities and tumor weights were compared between the LNCaP tumors generated in the presence of C57B prostate stromal cells engineered to express CTGF (*CTGF*) or vector control stromal cells (*Control*), in complete BD Matrigel conditions at day 13 postinoculation. *A,* microvessel density, as assessed by CD31-positive structures, counted by a blinded observer (n = 25 fields, five tumors for each group). *Statistically significant increase in tumor microvessel density for DRS tumors generated in the presence of stromal cells expressing CTGF ($P < 0.05$). *B,* tumor wet weight (n = 5). *Statistically significant increase in wet weights of CTGF-expressing tumors when compared with control tumors ($P < 0.01$). *C* and *D,* microvessel densities and tumor weight were compared between the LNCaP tumors generated in the presence of C57B cells engineered to express CTGF (*CTGF*) or vector control C57B stromal cells (*Control*), in the low growth factor modified matrix (GFR Matrigel/Vitrogen 100) conditions at day 13 postinoculation. *C,* microvessel density, as assessed by CD31-positive structures, counted by a blinded observer (n = 30 fields, six tumors for each group). *Statistically significant increase in tumor microvessel density for DRS tumors generated in the presence of stromal cells expressing CTGF ($P < 0.01$). *D,* tumor wet weight (n = 17 in the control and n = 18 in the CTGF experimental). *Statistically significant increase in wet weights of CTGF expression tumors when compared with control tumors ($P < 0.0001$).

Our studies and others have suggested that reactive stroma in carcinomas is an important process associated with early events in tumorigenesis, including the formation of a wound repair type of matrix and enhanced angiogenesis (1–3, 32). Reactive stroma is remarkably similar in most carcinomas. Typically, carcinoma-associated reactive stroma is composed of activated fibroblasts and myofibroblasts, characteristic of a wound repair–type stroma (1, 32, 35). A key feature of wound repair stroma is rapid and elevated angiogenesis. In wounding, platelet-released TGF-β1 and platelet-derived growth factor function to regulate stromal cell phenotype changes and to stimulate stromal cell migration, matrix production, and angiogenesis. TGF-β1 is overexpressed by cancer epithelial cells in most carcinomas, including prostate cancer (32, 35). Moreover, CTGF is TGF-β1 regulated in a diverse set of cell types, including human prostate stromal cells as reported here (15–19). In addition, CTGF has been shown to stimulate a wound repair type of stroma in several key studies and has been shown to mediate, in part, TGF-β1–induced matrix remodeling (20). Hence, it is important to determine whether CTGF mediates a TGF-β1–stimulated reactive stroma response in cancer and whether this reactive stroma is tumor promoting. Data reported here address this question directly and suggests that TGF-β1 stimulated CTGF expression in carcinoma-associated reactive stroma, promotes angiogenesis, and results in enhanced tumorigenesis.

It is becoming clearer that the classic regulators of wound repair play an important role in carcinoma-reactive stroma and CTGF biology. For example, both fibroblast growth factor-2 (FGF-2) and vascular endothelial growth factor have been reported to stimulate CTGF expression (25, 42). FGF-2 expression is also TGF-β1 regulated in fibroblasts from the prostate gland and other tissues (43, 44). Hypoxia will induce CTGF expression via a hypoxia-inducible factor-1α pathway (45). In addition, thrombin and plasma clotting actor VIIa also induce CTGF expression (46). Accordingly, several factors and conditions associated with wound repair are known to affect CTGF expression and many of these factors and conditions are likely to play a role in tumor-associated reactive stroma.

The specific mechanisms of how CTGF or closely related family members directly affect reactive stromal cells in the tumor microenvironment is not fully understood. It is known that both CTGF and Cyr61 promote fibroblast adhesion through integrin α6β1 and that this process requires cell surface heparan sulfate proteoglycans (47). Cry61 and CTGF also stimulated migration and proliferation of fibroblasts, as well as endothelial cells (24, 48). In addition, CTGF also affects matrix production and remodeling. For example, CTGF was shown to stimulate fibronectin expression via a p42/44 mitogen-activated protein kinase and phosphoinositide 3 kinase/protein kinase B pathway (49). It will be important to dissect key CTGF signaling pathways in reactive stroma associated with tumors. Key components of these mechanisms may be useful as targets of therapeutic approaches directed at the tumor microenvironment.

The DRS model described in this study brings the opportunity to use highly efficient gene delivery and stable gene integration

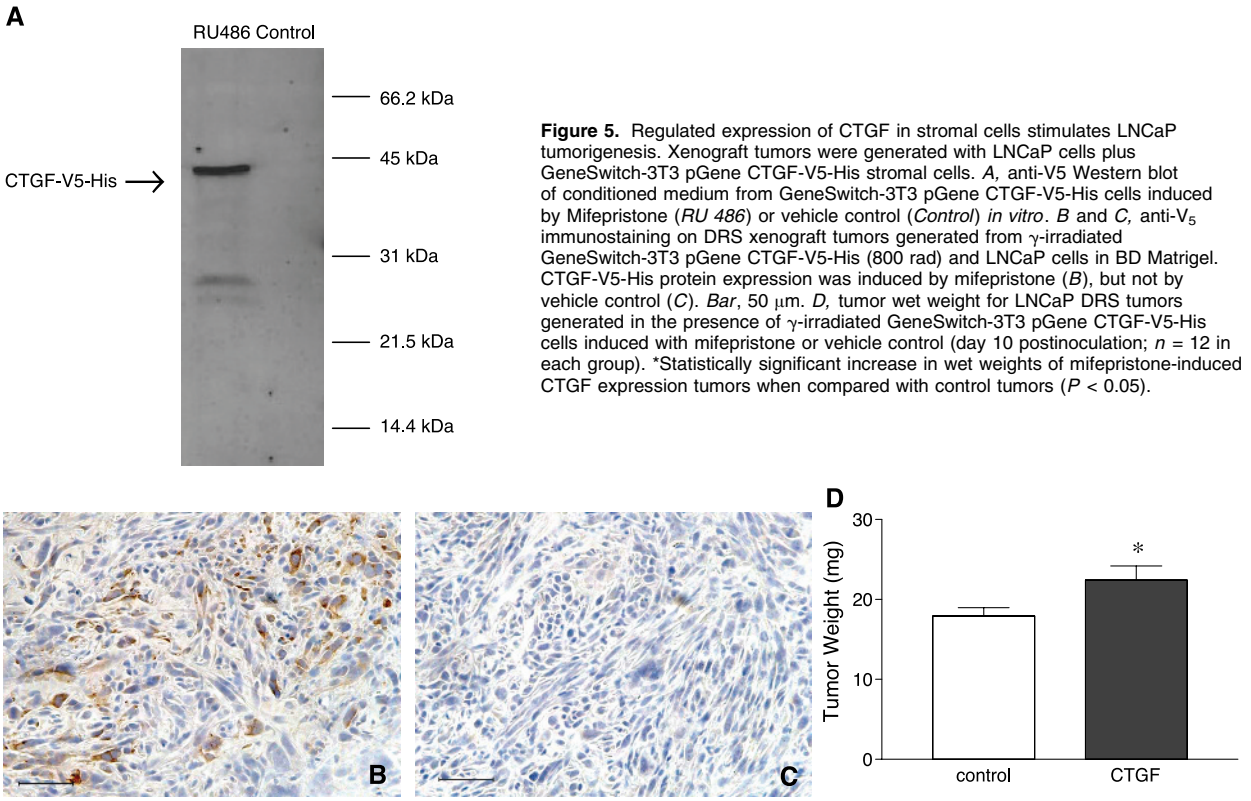

Figure 5. Regulated expression of CTGF in stromal cells stimulates LNCaP tumorigenesis. Xenograft tumors were generated with LNCaP cells plus GeneSwitch-3T3 pGene CTGF-V5-His stromal cells. *A,* anti-V5 Western blot of conditioned medium from GeneSwitch-3T3 pGene CTGF-V5-His cells induced by Mifepristone (*RU 486*) or vehicle control (*Control*) *in vitro*. *B* and *C,* anti-V_5 immunostaining on DRS xenograft tumors generated from γ-irradiated GeneSwitch-3T3 pGene CTGF-V5-His (800 rad) and LNCaP cells in BD Matrigel. CTGF-V5-His protein expression was induced by mifepristone (*B*), but not by vehicle control (*C*). *Bar*, 50 μm. *D,* tumor wet weight for LNCaP DRS tumors generated in the presence of γ-irradiated GeneSwitch-3T3 pGene CTGF-V5-His cells induced with mifepristone or vehicle control (day 10 postinoculation; $n = 12$ in each group). *Statistically significant increase in wet weights of mifepristone-induced CTGF expression tumors when compared with control tumors ($P < 0.05$).

of retroviral-infected mouse prostate stromal cell lines to study the roles of epithelial cell-stromal cell interactions in carcinoma tumorigenesis and progression. Accordingly, the DRS model has allowed for the ability to dissect out the roles of individual growth factors in the reactive stroma compartment of a tumor. Data reported here represent the first study to show that expression of CTGF in the tumor microenvironment stromal cells of an experimental epithelial cancer functions to stimulate angiogenesis and tumor growth.

Emerging data supports the concept that the reactive stromal tumor microenvironment functions to affect the rate of tumorigenesis in most epithelial carcinomas studied to date. Accordingly, it is likely that the biological components and specific mechanisms of reactive stroma can be used both as prognostic indicators and as targets of therapeutics. This study shows that CTFG is a TGF-β1–regulated and stromal-expressed factor that promotes tumorigenesis and is, therefore, a theoretical target for therapeutics focusing on tumor-associated reactive stroma biology.

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